PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classificati n 6 :
C12N 15/74, 15/62, 15/31, 1/21, C07K 14/33, 14/28, 14/245, 16/12, A61K 39/08, 39/106 // (C12N 1/21, C12R 1:63)

A1

(11) International Publication Number: WO 98/23763

(43) International Publication Date: 4 June 1998 (04.06.98)

(21) International Application Number: PCT/US97/21359

(22) International Filing Date: 25 November 1997 (25.11.97)

(30) Priority Data:
60/032,328 29 November 1996 (29.11.96) US

(71) Applicant: THE GENERAL HOSPITAL CORPORATION [US/US]; 55 Fruit Street, Boston, MA 02114 (US).

(72) Inventors: RYAN, Edward, T.; Apartment 2, 36 Irving Street, Boston, MA 02181 (US). CALDERWOOD, Stephen, B.; 6 Pilgrim Road, Wellesley, MA 02181 (US).

(74) Agents: ELLISON, Eldora, L. et al.; Fish & Richardson P.C., 601 Thirteenth Street, N.W., Washington, DC 20005 (US).

(81) Designated States: AU, CA, CN, JP, MX, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HETEROLOGOUS ANTIGENS IN LIVE CELL *V. CHOLERAE* STRAINS

(57) Abstract

Disclosed is a *V. cholerae* cell containing a DNA encoding a fusion protein that includes a heterologous antigenic polypeptide sequence such as a portion of *C. difficile* toxin A. The fusion protein also includes one of the following: (i) an *E. coli* hemolysin A subunit (which is coexpressed with sequences encoding hemolysin B and D subunits); (ii) a secretion signal sequence and cholera toxin A2 subunit (which is coexpressed with cholera toxin B subunit); or (iii) cholera toxin B subunit.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

HETEROLOGOUS ANTIGENS IN LIVE CELL *V. CHOLERAE* STRAINS

The field of the invention is genetically engineered live *V. cholerae* vaccine strains.

Statement as to Federally Sponsored Research

The work disclosed herein was supported in part by U.S. Public Health Service grants K08 AI01332 and K08 AI01386 from the National Institute of Allergy and Infectious Diseases. The U.S. Government therefore may have certain rights in the invention.

Background of the Invention

Microbial pathogens that infect mammals via mucosal surfaces have caused significant morbidity and mortality in the developing world. Of particular concern are pathogens that infect the gastrointestinal, respiratory, and genitourinary systems. Many proposed, conventional systemic immunization methods have been ineffective in protecting against infections that occur via such mucosal surfaces.

The Mucosal Immune System

The immune system is thought to be functionally separated into systemic and mucosal immune compartments (Czerkinsky et al., Cellular and Molecular, 1994, 1:37-44). The mucosal immune system represents the largest immunological organ in the body. Luminal antigens are processed via M (microfold) cells, which are specialized epithelial cells found in the gastrointestinal tract and are involved in the induction of a mucosal immune response (Neutra et al., Johnson LR, ed. Physiology of the Gastrointestinal Tract, Third Edition, 1994, 685-708). Antigen processing and presentation are followed by proliferation and differentiation of IgA-committed,

antigen-specific B lymphocytes that circulate via the bloodstream and populate the lamina propria of the upper respiratory, intestinal, and genitourinary tracts, as well as the salivary and mammary glands. In these effector sites, plasma cells produce antigen-specific IgA, which is then secreted across epithelial cells, acquiring secretory component in the process (Neutra et al., Johnson LR, ed., Physiology of the Gastrointestinal Tract, Third Edition, 1994, 685-708). Secretory component enhances resistance of these antibodies to proteolysis. The circulation of antigen-specific cells from one inductive site to multiple effector sites has led to the concept of a common mucosal immune system.

Vibrio cholerae

V. cholerae is a gram-negative bacterium that, in its wild-type state, causes severe, dehydrating and occasionally fatal diarrhea in humans. There are an estimated 5.5 million cases of cholera each year, resulting in greater than 100,000 deaths (Bull. W.H.O. 68:303-312, 1990). Over the last several decades, cholera has been considered to occur primarily in developing countries of Asia and Africa, but recently it has reached epidemic proportions in regions of South and Central America as well (Tauxe et al., J. Am. Med. Assn. 267:1388-1390, 1992; Swerdlow et al., J. Am. Med. Assn. 267:1495-1499, 1992).

Patients who recover from cholera infection have long-lasting, perhaps lifelong, immunity to reinfection (Levine et al., J. Infect. Dis. 143:818-820, 1981). The development of *V. cholerae* vaccines has focused on reproducing this naturally occurring immunity, but the conventional, parenteral, killed whole-cell vaccine preparation provides less than 50% protection from disease, for a duration of only 3 to 6 months (Saroso et

al., Bull. W.H.O. 56:619-627, 1978; Levine et al., Microbiol. Rev. 47:510-550, 1983).

The most important virulence factor for *V. cholerae* in causing clinical disease is cholera toxin, a protein complex consisting of one A subunit and five B subunits. An internal deletion of the gene encoding the A subunit of cholera toxin (*ctxA*) in the classical strain 0395 produces a strain (0395-N1) that is highly immunogenic in humans (Mekalanos, 1983, Nature 306:551-557; Herrington, 1988, J. Exp. Med. 168:1487-1492; Mekalanos, U.S. Patent No. 4,882,278, herein incorporated by reference).

Clostridium difficile

Clostridium difficile is the causative agent of pseudomembranous colitis and results in significant morbidity, mortality, and cost (Kelly et al., Gastroenterology, 1992, 102:35-40; Lyerly et al., Clin. Microbiol., 1988, Rev. 1:1-18; Mitty et al., Gastroenterologist, 1994, 2:61-69). *C. difficile* causes pseudomembranous colitis through the action of two large toxins, toxin A and toxin B, that modify Rho proteins with subsequent disruption of the actin cytoskeleton (Dillon et al., Infect. Immun., 1995, 63:1421-1426). Toxin A appears to initiate intestinal damage, to produce mucosal disruption, and to permit full cytotoxicity of toxin B (Lyerly et al., Clin. Microbiol., 1988, 1:1-18). The genes encoding toxin A and toxin B have been sequenced (Dove et al., 1990, Infect. and Immun. 58:480-488; von Esc et al., 1990, Gene 96:107-113; and von Eichel-Streiber et al., 1995, Mol. Microbiol. 17:313-321 (GenBank Accession No. Z23277)). The carboxy terminal third of toxin A, which is approximately 800 amino acids in length, is essential for toxin binding to trisaccharide receptors on human intestinal epithelial

cells (Dove et al., 1990, Infect. and Immun. 58:480-488; Krivan et al., Infect. Immun., 1986, 53:573-581; Lyerly et al., Clin. Microbiol., 1988, 1:1-18; Sauerborn et al., Nucl. Acids Res., 1990, 18:1629-1630; Tucker et al., Infect. Immun., 1991, 59:73-78; von Eichel-Streiber et al., Gene, 1990, 96:107-113). Antibodies directed against toxin A prevent toxin binding, neutralize secretory and inflammatory effects, and limit or prevent clinical disease (Allo et al., Gastroenterology, 1979, 76:351-355; Corthier et al., Infect. Immun., 1991, 59:1192-1195; Johnson et al., J. Immunol., 1993, 150:117A Abstract #657; Ketley et al., J. Med. Microbiol., 1987, 24:41-52; Kim et al., Infect. Immun., 1987, 55:2984-2992; Leung et al., J. Pediatr., 1991, 118:633-637; Warny et al., Infect. Immun., 1994, 62:384-389). Antibodies specifically directed against the carboxy terminus of toxin A have been shown to prevent holotoxin binding and abrogate subsequent cytotoxic events (Corthier et al., Infect. Immun., 1991, 59:1192-1195; Frey et al., Infect. Immun., 1992, 60:2488-2492; Lyerly et al., Clin. Microbiol., 1988, Rev. 1:1-18; Wren et al., Infect. Immun., 1991, 59:3151-3155).

Summary of the Invention

As described in detail below, it has now been found that a potent immune response against a heterologous antigen can be stimulated in an animal by recombinant *V. cholerae*-vaccine cells that express *E. coli* hemolysin B (HlyB) and hemolysin D (HlyD) subunits along with a fusion polypeptide that includes a heterologous antigen (e.g., all or a portion of *C. difficile* toxin A or toxin B) fused to *E. coli* hemolysin subunit A (HlyA) or a portion thereof.

An alternative means for expressing an heterologous antigen in *V. cholerae* in a way that would

induce a protective immune response is by expressing both cholera toxin B subunit (either naturally or recombinantly) and a fusion polypeptide that includes a secretory signal sequence, a heterologous antigen (e.g., all or a portion of *C. difficile* toxin A or toxin B), and cholera toxin A2 subunit.

Also described herein is a third type of recombinant *V. cholerae* strain. In this case, the cells are genetically engineered to express a fusion polypeptide that includes cholera toxin B subunit fused to an antigenic portion of *C. difficile* toxin A or toxin B subunit. In a preferred *V. cholerae* cell, cholera toxin B is expressed from a *tac* promoter on the plasmid pETR1. To produce pETR1, *ctxB*, including the upstream Shine-Dalgarno sequence, was recovered from C6709 (Taylor et al., 1994, J. Inf. Dis. 170:1518-1523) by recombinant, mutagenic PCR, with introduction of a unique *Nhe*I site two amino acids downstream of the coding sequence for the CtxB leader peptide, and the resulting fragment was introduced between the *Eco*RI and *Pst*I sites of pKK223-3. Cholera toxin B expressed from pETR1 is secreted to the supernatant and recognized in ELISA assays (data not shown).

Without being bound to any particular theory, it is believed that each of the *V. cholerae* strains described above allows secretion of the fusion polypeptide from the vaccine strain into the gastrointestinal tract of an immunized animal. Fusion polypeptides that include the *E. coli* hemolysin A subunit are thought to be secreted via an *E. coli* hemolysin transport system that is reconstituted in the *V. cholerae* cell. Once the heterologous antigens are present in the lumen of the immunized animal, the antigens are processed via "M" (microfold) cells, which participate in the induction of a mucosal immune response (Neutra et al.,

Secretory Immunoglobulin A. Induction, Biogenesis, and Function. *In*: Johnson, *ed*. Physiology of the Gastrointestinal Tract. New York: Raven Press, 1994:685-708).

The invention thus provides the above-described *V. cholerae* cells (also referred to herein as "vaccine strains"), which can be admixed with a pharmaceutically acceptable excipient in formulating *V. cholerae*-based vaccines. Also included within the invention are methods for inducing an immune response in an animal (e.g., a human) by administering to the animal one or more of the aforementioned *V. cholerae*-based vaccine strains. If desired, the *V. cholerae*-based vaccines can be administered to the animal along with an immunoadjuvant. For example, purified detoxified proteins such as detoxified cholera toxin and/or detoxified heat labile enterotoxin can be given orally as detoxified immunoadjuvants (*see*, e.g., Clements et al., 1995, Infect. Immun. 63:1617-1627; Rappuloi et al., 1994, Infect. Immun. 63:2356-2360). As an alternative to orally administering the detoxified immunoadjuvant to an animal, conventional molecular biology techniques can be used to express the detoxified immunoadjuvant from the *V. cholerae* vaccine strain administered to the animal. "Detoxified immunoadjuvants" are bacterial toxins that have been mutagenized such that they are not toxic to the cell to which they are delivered or in which they are expressed.

A variety of *V. cholerae* strains can be used as "background" strains in engineering the *V. cholerae* vaccine strains of the invention. Preferred background strains include *V. cholerae*-O1 strain 569B, *V. cholerae*-01 strain 0395, and *V. cholerae-0139* strain Bengal 2 and their vaccine derivatives. The classical *V. cholerae*-O1

CA401 and El Tor O1 strain Bahrain 2 and C6709 (with its derivative Peru2) are less preferred.

"Heterologous antigenic polypeptide" is defined as an antigenic polypeptide (e.g., a naturally-occurring polypeptide) that is not naturally expressed by *V. cholerae*. It is preferably a polypeptide that is naturally expressed by an infectious organism (other than *V. cholerae*), and which induces an antigenic response in an animal (preferably a mammal such as a human, non-human primate, cow, horse, sheep, goat, pig, dog, cat, rabbit, rat, mouse, guinea pig, or hamster). If desired, the heterologous antigenic polypeptide can be a fusion polypeptide, a fragment of a protein, or a naturally-occurring or synthetic epitope.

Typically, the infectious organism is a bacterium (e.g., *Clostridium difficile*), a virus, or a eukaryotic parasite. Preferred heterologous antigenic polypeptides include, e.g., an immunogenic bacterial toxin such as *C. difficile* toxin A or B, Shiga toxin, diphtheria toxin, *Pseudomonas* exotoxin A, pertussis toxin, tetanus toxin, anthrax toxin, one of the *E. coli* heat-labile toxins (LTs), one of the *E. coli* heat-stable toxins (STs), or one of the *E. coli* Shiga-like toxins; an OSP (Outer Surface Protein) of *Borrelia burgdorferi*; an immunogenic, nontoxic bacterial protein such as a colonization factor of diarrheogenic *E. coli*, a colonization factor of *Bordetella pertussis*, a pilin of uropathogenic *E. coli*, or a pilin of *Neisseria gonorrhoeae*; an immunogenic viral surface protein from a virus such as human immunodeficiency virus (HIV), any of the Herpes viruses (e.g., Herpes simplex virus or Epstein-Barr virus), influenza virus, poliomyelitis virus, measles virus, mumps virus, rubella virus, rotavirus, respiratory syncytial virus, adenovirus, or papilloma virus; or an immunogenic protein derived from a eukaryotic parasite,

such as the causative agent for malaria, pneumocystis pneumonia, or toxoplasmosis. One preferred example of such a protein is a malarial circumsporozoite protein. Where the heterologous antigenic polypeptide is a toxin (e.g., *C. difficile* toxin A or toxin B), a non-toxic portion of the toxin is included in the fusion polypeptide, while toxic portions are excluded.

Where the heterologous antigenic polypeptide is derived from *C. difficile* toxin A, the portion used in the fusion polypeptide is preferably a portion (e.g., at least 8 amino acids, and preferably at least 10) or all of the repeating peptide domain near the carboxy terminus of toxin A. When an antigenic portion of *C. difficile* toxin A or toxin B is fused to cholera toxin B subunit, the antigenic portion typically is 8-100 (preferably 8-20, and more preferably 10) amino acids in length. A preferred heterologous antigen derived from *C. difficile* toxin A has the amino acid sequence TIDGKKYYFN (SEQ ID NO:1). Where a heterologous antigenic polypeptide is fused to a secretion signal sequence and the cholera toxin A2 subunit, the antigenic polypeptide is typically 20 to 100, preferably 30 to 50, amino acids in length. When this heterologous antigenic polypeptide is derived from *C. difficile*, a 44 amino acid peptide from the peptide repeat sequence of toxin A provides a preferred heterologous antigen. This 44 amino acid epitope of *C. difficile* is encoded on the plasmid pCD11 (Price et al., 1987, Curr. Microbiol. 16:55-60), and is specifically bound by the monoclonal antibody PCG-4 (Dove et al., 1990, Infect. Immun. 58:480-488). Preferred secretion signal sequences for use in the invention are the secretion sequence of *E. coli* HlyA, of *E. coli* heat labile enterotoxin B subunit, of cholera toxin A subunit, and of *pelB*.

If desired, the nucleic acid encoding the fusion polypeptide can be functionally (or "operatively") linked to any promoter which functions in *V. cholerae* and permits expression at an acceptable level *in vivo*. Construction of such a functional linkage can be accomplished as described in detail below, or generally, using standard methods, by locating the desired promoter sequence sufficiently near to (and typically, though not necessarily, just upstream of) the promoterless sequence encoding the fusion polypeptide to permit the desired promoter sequence to control expression of the sequence encoding the fusion polypeptide. Functional siting of promoter sequences is well within the abilities of one of ordinary skill in the art of prokaryotic gene expression. Where the promoter naturally controls the expression of a *V. cholerae* virulence factor that is not essential for growth of the cell (e.g., an iron-regulated promoter such as that of *irgA*), the sequence encoding that virulence factor will preferably be deleted or otherwise mutated to prevent expression of a biologically active form of that virulence factor.

Preferably, the natural *ctxA* locus on the *V. cholerae* chromosome will be deleted or otherwise inactivated, so that biologically active cholera toxin cannot be expressed from the chromosome. Such deletions, mutations and insertions can readily be carried out by one of ordinary skill using the methods described herein, or other well-known, standard techniques. In preferred embodiments, the *ctxA* deletion is identical to that of strain 0395-N1 (Mekalanos, U.S. Patent No. 4,882,278).

Construction of a promoter sequence adjacent to a sequence encoding a fusion polypeptide containing a given heterologous antigen-encoding sequence, and insertion of the resulting construct into a *V. cholerae* genome or a

plasmid, is readily accomplished by one of ordinary skill.

The sequences encoding the desired polypeptides (e.g., the fusion polypeptides, HlyA, HlyB, HlyD, and cholera toxin B subunit) can be expressed in *V. cholerae* cells by integrating the sequences into the *V. cholerae* genome or by carrying the sequences on a plasmid that is introduced into the *V. cholerae* cell. If desired, a given *V. cholerae* cell can express one or more of these sequences from a plasmid, or the sequence can be integrated into the cell's chromosome. Conventional molecular biology techniques can be used to produce recombinant *V. cholerae* strains and plasmids for use in the invention. Methods for *in vivo* marker exchange, to introduce genes into the *V. cholerae* chromosome, are known to those of skill in the art (see, e.g., Butterton et al., 1995, Infect. Immun. 63:2689-2696). If desired, the *V. cholerae* strains and plasmids can contain a balanced lethal mutation (i.e., a lethal chromosomal mutation that is balanced by a gene expressed from the plasmid to overcome lethality). Such balanced lethal mutations are particularly useful when using the vaccine strains of the invention to express heterologous antigens in humans.

The *V. cholerae* cells of the invention can be said to define a vaccine strain useful, when combined with a pharmaceutically acceptable excipient suitable for oral administration, as a live-cell vaccine. Administration of such a vaccine to an animal (e.g., a human or other mammal) will provoke immunity not only to *V. cholerae*, but also to the organism from which the heterologous antigen is derived; it thus serves as a bivalent vaccine. An exemplary vaccine utilizes a *V. cholerae* strain genetically engineered to express *E. coli* HlyB and HlyD and a fusion polypeptide that includes (a) an antigenic,

non-toxic portion of *C. difficile* toxin A or toxin B and (b) *E. coli* HlyA. This vaccine strain is described in detail below. Of course, the bacterial strain of the invention could be engineered to encode several heterologous antigens, each linked to an identical or different promoter, to produce a multivalent vaccine effective for simultaneously inducing immunity against a number of antigens or infectious diseases. In such strains, the various heterologous antigens form part of one or more fusion polypeptides that are expressed in the *V. cholerae* cells.

The invention offers several advantages. *V. cholerae* is a non-invasive organism that attaches selectively to intestinal M cells. Thus, the antigens are presented directly to underlying lymphoid tissues, permitting strong and long-lasting mucosal immune responses. *V. cholerae* colonizes human intestinal tissues for 7-14 days, thereby allowing for repeated antigenic presentation after a single administration of the vaccine. In addition, the vaccines of the invention can be administered orally. Thus, several problems inherent in parenteral immunization are avoided, such as the use of needles, the need for strict refrigeration of the vaccine, and the need for specially trained personnel to administer the vaccine by injection. Other features and advantages will be apparent from the detailed description provided below, and from the claims.

Brief Description of the Drawings

Fig. 1A is a graph depicting percentage of total toxin A protein in supernatant, cytoplasmic, periplasm, and membrane-associated fractions. Fig. 1B is a graph representing the percentage of total toxin A protein and β-galactosidase activity in supernatant fractions. Fig. 1C is a graph representing the percentage of total toxin

A protein in supernatant fractions of various *V. cholerae* strains (see Table 1 and text for details). For each graph, results are reported as geometric means, and bars depict standard errors of the mean (SEM) for each group. +, $p<0.01$, compared to the O395-NT (pMOhly1) control.

Figs. 2A and 2B are graphs illustrating ileal colonization after oral inoculation with *V. cholerae* O395-NT (pETR14) in rabbits treated with water that was not supplemented with ampicillin (Fig. 2A) or water supplemented with 1 mg/ml of ampicillin (Fig. 2B). Solid bars represent the geometric means titers of recovered *V. cholerae* vector, O395-NT. Striped bars represent the titers of recovered *V. cholerae* vector still containing plasmid pETR14. Bars represent SEM for each group.

Fig. 3 is a graph showing geometric mean titers (GMT) of vibriocidal antibody responses on days 15 and 28 following inoculation of rabbits with *V. cholerae* vector strains with or without 15 μg of cholera toxin (CT) orally as an immunoadjuvant. Bars represent SEM for each group.

Fig. 4 is a graph illustrating serum IgG antibody responses against *C. difficile* toxin A on days 14, 21, and 28, in rabbits that received two inoculations of the indicated strains on days 0 and 14, with or without 15μg of CT orally. Results are reported as end GMTs; bars depict SEM for each group. +, $p<0.01$; *, $p<0.05$, compared to group receiving O395-NT (pMOhly1).

Fig. 5 is a graph representing bile IgA antibody responses against *C. difficile* toxin A on day 28 in rabbits that received two oral inoculations of the indicated strains on days 0 and 14, with or without 15μg of CT orally. Results were determined by a kinetic ELISA reading; squares represent data points (mOD/min) from individual animals. The geometric means plus SEMs fcr each group are shown.

Figs. 6A-6D are a series of graphs illustrating protection against *C. difficile* toxin A in an ileal loop challenge assay, following oral inoculation with various strains on days 0 and 14, with or without 15μg of CT administered orally. Fluid secretory responses in ileal loops (weight/length) were measured 12 hours after intraluminal administration of PBS/BSA (Fig. 6A), 10μg of cholera toxin (Fig. 6B), 1μg of *C. difficile* toxin A (Fig. 6C), or 5μg of toxin A (Fig. 6D). Results are reported as the geometric means of the secretory response; bars represent the SEM for each group. +, $p<0.01$; *, $p<0.05$, compared to vaccination with 0395-NT (pMOhly1).

Figs. 7A-7E are a series of photographs of histological sections of ilea recovered from vaccinated and control animals. Fig. 7A illustrates the results obtained following intraluminal challenge with PBS/BSA. Figs. 7B and 7D illustrate the results obtained following intraluminal challenge with 1 μg of *C. difficile* toxin A. Figs. 7C and 7E illustrate the results obtained following intraluminal challenge with 5μg *C. difficile* toxin A. Intestinal segments shown in Figs. 7A, 7B and 7C are from control animals that received *V. cholerae* 0395-NT(pMOhly1). Intestinal segments shown in Figs. 7D and 7E are from a vaccinated animal that received *V. cholerae* 0395-NT (pETR14) expressing toxin A-HlyA. Magnification is 40X on hematoxylin-and-eosin stained sections of paraffin-imbedded tissues.

Detailed Description

In the experiments described below in Example 1, a non-toxic, 720 amino acid portion of *C. difficile* toxin A subunit was used as a model heterologous antigen. This heterologous antigen was fused to an *E. coli* HlyA secretion signal sequence, and the resulting fusion

polypeptide was co-expressed in *V. cholerae* with HlyB and HlyD. The fusion polypeptide was properly exported to the extracellular environment of *V. cholerae*, presumably by a mechanism that involves HlyB and HlyD. It was recognized by antibodies directed against *C. difficile* toxin A. When this vaccine strain was administered to rabbits, it successfully induced a protective mucosal and systemic immune response against *C. difficile* toxin A.

Examples 2 and 3 describe alternative strategies for expressing *C. difficile* toxin A epitopes in *V. cholerae*, for use in a bivalent vaccine. Example 4 describes use of such vaccines. It is noted that the *V. cholerae* cells described herein could also be used for *in vitro* production of the described fusion polypeptides for use in any *in vitro* assay (e.g., an immunoassay) requiring such a polypeptide. It is also noted that multiple plasmids encoding different fusion polypeptides with different heterologous antigenic peptide sequences could be introduced into a single *V. cholerae* strain, to produce a multivalent vaccine.

Those skilled in the art will recognize that alternative reagents and methods can be substituted for those described herein. These examples are meant to be illustrative, not limiting, as the metes and bounds of the invention are defined by the claims.

EXAMPLE 1

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

The bacterial strains and plasmids are summarized in Table 1. All strains were maintained at -70°C in Luria-Bertani broth (LB) medium (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., 1989) containing 15% glycerol. Streptomycin (100 μg/ml), tetracycline (25 μg/ml), and ampicillin (100 μg/ml) were added as appropriate. Cultures were grown at 37°C with aeration. Quantitative cultures were grown on thiosulfate-citrate-bile salts-sucrose (TCBS) plates or LB-agar plates containing appropriate antibiotics.

Table 1. Bacterial strains and plasmids

Strain or Plasmid	Relevant genotype or phenotype	Source or reference
V. cholerae strains		
O395-NT	Serotype O1, classical, Ogawa, O395 Δ*ctxAB*, Km^r, Sm^r	*a*
O395-N1	O1, classical, Ogawa, O395 Δ*ctxA*, Sm^r	*a*
569B	O1, classical, Inaba, wild-type	*b*
CA401	O1, classical, Inaba, wild-type, Sm^r	*c*
C6709	O1, El Tor, Inaba, wild-type	*d, e*
Peru2	C6709 Δ*attRS1*, Sm^r	*d, e*
Bahrain2	O1, El Tor, Ogawa, E7946 Δ*attRS1*, Sm^r	*e*
Bengal2	Serotype O139, M010, Δ*attRS1*, Sm^r	*f*
E. coli strains		
JM105	*thi*, *rpsL*, *endA*, *sbcB*15, *hsdR*4, *sup*E, Δ(*lac-proAB*), F' [*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ* ΔM15]; Sm^r	*g*
Plasmids		

pCD11	4.7 kbp *Pst*I fragment of *C. difficile* 10463 chromosomal DNA encoding the nontoxic carboxy terminal 2/3 of toxin A, cloned in pBR322; fragment contains an internal *Pst*I site; Tet^r	*h*
pMOhly1	plasmid encoding the hemolysin operon of *E. coli*, with internal deletion of *hlyA* such that the nucleotides for the amino terminal 34 amino acids are fused with the carboxy terminal 61 amino acid HlyA secretion signal at unique *Nsi*I site; Ap^r	*i*
pETR14	2.1 kbp *Pst*I fragment from 3' end of insert of pCD11 encoding the nontoxic carboxy terminal 1/3 of *C. difficile* toxin A, inserted in the *Nsi*I site of pMOhly1, in-frame between amino and carboxy termini of HlyA, Ap^r	*j*

Ap^r, ampicillin resistant; Sm^r, streptomycin resistant; Tet^r, tetracycline resistant

a: Mekalanos et al., 1983, Nature 306:551-557
b: Mekalanos, 1983, Cell 35:253-263
c: Sigel and Payne, 1982, J. Bacteriol. 150:148-155
d: Taylor et al., 1994, J. Infect. Dis. 170:1518-1523
e: Butterton et al., 1995, Infect. Immun. 63:2689-2696
f: Waldor and Mekalanos, 1994, J. Infect. Dis. 170:278-293
g: Pharmacia P-L Bio-chemical Inc., Milwaukee, WI
h: Price et al., 1987, Curr. Microbiol. 16:55-60
i: Gentschev et al., 1995, Infect. Immun. 63:4202-4205
j: Described herein

Genetic methods.

Isolation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis, and Southern hybridization of DNA separated by electrophoresis were performed according to standard molecular biological techniques (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., 1989). DNA sequencing was

Laboratory Manual, 2nd Ed., 1989). DNA sequencing was performed using ABI Prism DiTerminator Cycle™ sequencing with AmpliTaq™ DNA polymerase FS employing an ABI377 DNA Sequencer (Perkin Elmer Applied Biosystems Division, Foster City, CA).

Plasmids were transformed into *E. coli* strains by standard techniques. Plasmids were electroporated into *V. cholerae* using a Gene Pulser™ (Bio-Rad Laboratories, Richmond, CA) following the manufacturers' protocol, and modified for electroporation into *V. cholerae* as previously described (Goldberg et al., Proc. Natl. Acad. Sci. USA, 1991, 88:1125-1129). Electroporation conditions were 2,500 V at 25-μF capacitance, producing time constants of 4.6-4.8 ms.

DNA restriction endonucleases, T4 DNA ligase, and calf intestinal alkaline phosphatase were used according to conventional methods, as recommended by the manufacturers. Restriction digested DNA fragments were separated on 1% agarose gels; the desired DNA fragments were cut from the gels under UV illumination and the DNA was recovered with GenElute Agarose Spin Columns (Supelco Inc., Bellefonte, PA).

Plasmid construction.

Plasmid pETR14 was constructed by placing the 2.1 kbp *Pst*I fragment of pCD11 in-frame into the compatible-overhang *Nsi*I site of pMOhly1 treated with calf intestinal alkaline phosphatase. The ligation product was transformed into *E. coli* JM105 and confirmed by restriction enzyme digestion and sequence analysis. pETR14 was electroporated into the *V. cholerae* strains of interest and successful electroporation was confirmed by restriction enzyme digestion.

Characterization of Toxin A-HlyA.

Supernatant and intracellular fractions were produced using conventional methods. Overnight cultures (3 ml) were centrifuged at 14,000 rpm (Eppendorf Centrifuge 5415C; Brinkmann Instruments, Inc., Westbury, NY) for 10 minutes. Supernatants were recovered and passed through a 0.45 μm sterile ACRODISC™ filter (Gelman Sciences, Ann Arbor, MI). Recovered cell pellets were washed in 1 M Tris-HCl, pH 8.0, then centrifuged for 5 minutes at 14,000 rpm, and the resulting pellets were recovered. Cell pellets were frozen at -70°C for 30 minutes, thawed and resuspended in 1 M Tris-HCl, pH 8.0. The resuspended cell pellets were sonicated for three 5-second bursts at 60% probe intensity (Biosonik Bronwill Scientific, Rochester, NY). Freeze-thawed sonicates were spun at 14,000 rpm for 30 minutes at 4° C, and the supernatant was recovered as the intracellular fraction. Supernatant and intracellular preps were used immediately or stored at -70° C.

Immunodetection of Toxin A-HlyA.

The amount of toxin A-HlyA present in intracellular and supernatant fractions was quantified with an enzyme linked immunosorbant assay (ELISA). Briefly, serial dilutions (undiluted - 1:2,185) in phosphate buffered saline-0.05% Tween 20 (PBS-T) of intracellular or supernatant samples were applied to 96-well microtiter plates. The wells of the microtiter plates were previously coated with mouse anti-*C. difficile* toxin A monoclonal antibody PCG-4 (0.4 mg/ml; TechLab, Inc., Blacksburg, VA) in 50 mM carbonate buffer, pH 9.6, and blocked with phosphate buffered saline-1% bovine serum albumin (PBS/BSA) (Sigma Chemical Co., St. Louis, MO). The plates were incubated at room temperature overnight and then washed in PBS-T. A

1:4,000 dilution of polyclonal goat anti-*C. difficile* toxin A antibody (TechLab) in PBS-T was then applied. After incubating the plates at room temperature for 12 hours and washing the plates with PBS-T, a 1:6,000 rabbit anti-goat IgG antibody horse radish peroxidase conjugate (Southern Biotechnology Associates, Inc., Birmingham, AL) was applied for overnight room temperature incubation. Plates were then washed, and the reactions were developed with 2,2-azino-*bis* (3-ethyl benzthiazoline)-6-sulfonic acid (ABTS; Sigma) with 0.1% H_2O_2 (Sigma). The optical density at 405 nm was read in a Vmax Microplate Reader (Molecular Devices Corp., Sunnyvale, CA). Measured optical densities were compared with a standard curve generated by serial dilutions of purified *C. difficile* toxin A (TechLab).

Measurement of β-galactosidase activity.

β-galactosidase activities in undiluted and 1:10 dilutions of supernatant and intracellular fractions of various *V. cholerae* strains were measured using conventional methods (Sambrook et al., 1989, Molecular cloning: a laboratory manual, 2nd Ed.) The percentages of β-galactosidase activities in intracellular versus supernatant fractions were compared with percentages of toxin A protein measured in parallel.

Inoculation of rabbits.

Orogastric colonization of rabbits was performed as previously described (Cray et al., Infect. Immun., 1983, 41:735-741). *V. cholerae* strains were grown overnight in LB medium, pelleted, washed in PBS, and resuspended in fresh LB medium to a final concentration of 10^{10} cfu per ml (approximately 80% containing plasmid). Male New Zealand white rabbits (approximately 2.5 kg; 9-11 weeks old) were fasted overnight and sedated. Gastric

acid was neutralized with 50 mg cimetidine per kg IV at time 0. At 20 minutes, 10 ml of a (1:1) 5% $NaHCO_3$:*V. cholerae* inoculum in LB was given using a gastric tube. At 60 minutes, 1 ml of tincture of opium was given intraperitoneally. For rabbits receiving cholera holotoxin (CT) as an immunoadjuvant, 15 mg of CT (List Biological Laboratories Inc., Campbell, CA) was added for each oral inoculum. Vaccinated rabbits received two oral inocula; the first on day 0, and the second on day 14. Blood was drawn on days 0, 14, 21, and 28. Bile was obtained on day 28. Three rabbits were inoculated with 0395-NT (pMOhly1) with 15 mg CT per inoculum; three rabbits were inoculated with 0395-NT (pETR14) without CT, and three rabbits were inoculated with 0395-NT (pETR14) with 15 mg of CT per inoculum.

To judge intestinal colonization of *V. cholerae* 0395-NT (pETR14) and plasmid retention, a separate cohort of rabbits was orally inoculated once. Washed ilea from these rabbits were quantitatively examined for the presence of pETR14 on serial days as previously described (Cray et al., Infect. Immun., 1983, 41:735-741; Pierce et al., Infect. Immun., 1988, 56:142-148; Pierce et al., Infect. Immun., 1985, 813-816). Rabbits were inoculated as described above with 10^{10} cfu of 0395-NT (pETR14). The plasmid pETR14 contains an ampicillin resistance gene. Four rabbits received normal water and two rabbits received water containing ampicillin (1 mg/ml). Beginning on day 2, rabbits were appropriately anesthetized and then sacrificed with Fatal-Plus (Vortech Pharmaceuticals, Dearborn, MI) on serial days. A 10 cm segment of ileum (beginning 10 cm proximal to the mesoappendix and moving cephalad) was removed, opened, washed in PBS, weighed, and homogenized (Tissue Grinder, Corning Inc., Corning, NY) in LB. The removed ileum was then quantitatively cultured on LB medium containing

streptomycin (Cray et al., Infect. Immun., 1983, 41:735-741; Pierce et al., Infect. Immun., 1988, 56:142-148; Pierce et al., Infect. Immun., 1985, 813-816). After overnight incubation, colonies were replica-plated on thiosulfate-citrate-bile salts-sucrose plates and on LB medium containing ampicillin. DNA from representative colonies growing on LB medium containing ampicillin was isolated in order to confirm the presence of pETR14.

Rabbit serum vibriocidal antibodies.

Serum vibriocidal antibody titers were measured in a microassay as follows. The endogenous complement activity of test sera was inactivated by heating sera at 56°C for one hour. Fifty μl serial 2-fold dilutions (1:25-1:25,600) of test sera and PBS were placed in wells of sterile 96-well tissue culture plates. Fifty μl of a $1x10^8$ cfu/ml culture of *V. cholerae* O395-NT in PBS with 22% guinea pig complement (Gibco BRL Life Technologies, Gaithersburg, MD) was added to the serum dilution and incubated for 1 hour at 37°C. A 150 μl aliquot of brain heart infusion broth (Difco Laboratories, Detroit, MI) was added to each well and plates were incubated for 2.5 hours at 37°C. The optical density at 600 nm was then measured. Titers were determined by determining the dilution of serum causing a 50% reduction in optical density compared with wells containing preimmune (day 0) serum.

Rabbit serum and biliary antibodies to Toxin A-HlyA.

Microtiter plates (96-well) were coated with 100 ng/well of mouse monoclonal anti-*C. difficile* toxin A antibody PCG-4 (TechLab). After an overnight room-temperature incubation followed by washing, purified *C. difficile* toxin A in carbonate buffer, pH 9.6, (100 ng/well; TechLab) was added. Plates were incubated

overnight, washed, and blocked with PBS/BSA. Duplicate serial dilutions of sera (1:25-1:492,075) from days 0, 14, 21, and 28 were incubated overnight. A 1:1,000 goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) in PBS/T/2% dried milk was added and plates were incubated overnight. Reactions were developed with 2 mg/ml of *p*-nitrophenyl phosphate (Amnesco Inc., Solon, OH) diluted in 1 M Tris-HCl, pH 8.0. The optical density at 405 nm was kinetically measured. Plates were read for 5 minutes at 19 second intervals and the maximum slope was reported as milli-optical density units per minute (mOD-min). End dilutions were defined as the highest dilution of day 14, 21, and 28 sera for which the kinetic reading was statistically greater ($p<0.05$) than the kinetic reading for preimmune (day 0) serum.

Serum IgA anti-toxin A-HlyA was similarly examined with a 1:1,000 goat anti-serum against rabbit IgA antibody (α chain specific; Sigma) followed by a 1:1,000 rabbit anti-goat IgG alkaline phosphatase conjugate (Sigma). Reactions were developed and read as described above.

Anti-toxin A-HlyA IgA antibody response in bile was measured in quadruplicate at a 1:100 dilution in PBS-T in 96-well microtiter assay plates previously coated with PCG-4 and toxin A. A 1:1,000 goat anti-serum against rabbit IgA (Sigma) in PBS/T/milk (2%) was followed by a 1:2,000 rabbit anti-goat IgG alkaline phosphatase conjugate. Plates were developed and read as described above.

Rabbit ileal loop Toxin A challenge assay.

Rabbit ileal loop procedures were performed as previously described (Acheson et al., Infect. Immun., 1996, 64:355-357; Formal et al., Br. J. Exp. Path., 1961, 42:504-510; Ketley et al., J. Med. Microbiol., 1987,

24:41-52; Lima et al., Infect. Immun., 1988, 56:582-588; Mitchell et al., Gut, 1986, 27:78-85). On day 28, rabbits were anesthetized. After shaving and preparing the rabbit, the rabbit's abdomen was opened with a single midline incision. Intestines were mobilized, and the duodenum was tied off. Serial 10 cm distal small intestinal segments were tied off. Short, 2 cm intervening intestinal segments were used as spacers between the 10 cm intestine segments. Mesenteric vessels and vascular arcades were avoided. A 1 ml aliquot of 1 μg *C. difficile* toxin A (TechLab) or 5 μg toxin A was instilled intraluminally into additional 10 cm intestinal segments. Installation of 1 ml of PBS/BSA or 1 ml cholera toxin (10 μg; List) into additional 10 cm intestinal segments were employed as negative and positive controls, respectively. All installations were performed in duplicate in each animal. The intestines were then replaced within the abdominal cavity and the incision closed. Animals were returned to their cages and comfortably maintained. After 12 hours, animals were appropriately anesthetized and then sacrificed. Each ileal loop segment was removed, inspected, weighed, and its length measured. The weight to length (gm/cm) ratios were calculated for each intestinal segment. Intestines were preserved in 10% formalin and histological examination was performed.

Statistics and graphics.

Data were plotted using CA-Cricket Graph Software (Computer Associates, Garden City, NY) and statistical significance was analyzed with a two-tailed *t*-test for the comparison of means.

RESULTS

Construction of pETR14.

A 2160 bp *Pst*I fragment encoding the carboxy terminus of *C. difficile* toxin A was removed from pCD11 (Price et al., Curr. Microbiol., 1987, 16:55-60) and ligated to the compatible *Nsi*I site of pMOhly1 (Gentschev et al., Infect. Immun., 1995, 63:4202-4205) such that the toxin A sequence was in-frame with the residual upstream (encoding for 34 amino terminal HlyA amino acids) and downstream (encoding for 61 carboxy terminal HlyA amino acids) sequences of *hlyA* to create plasmid pETR14. This plasmid also contained *E. coli hlyB* and *hlyD*. pETR14 was introduced into *E. coli* JM105 and various *V. cholerae* strains including *V. cholerae* O395-NT. O395-NT is a vaccine strain derived from classical *V. cholerae* O395, from which both copies of *ctx* have been removed (Mekalanos et al., Nature, 1983, 306:551-557).

Characterization of the Toxin A-HlyA Fusion Polypeptide.

Examination of overnight cultures disclosed that the fusion polypeptide was produced by *E. coli* JM105 (pETR14) at approximately 30-300 ng/ml/OD_{600}, and the fusion polypeptide was produced by *V. cholerae* O395-NT (pETR14) at 5-50 ng/ml/OD_{600}. An examination of supernatants of whole cell fractions revealed that over 90% of toxin A-HlyA was localized in the supernatant in JM105 (pETR14), and over 80% was localized in the supernatant in O395-NT (pETR14) (Fig. 1A). Simultaneous examination of β-galactosidase activity on whole cell and supernatant fractions revealed that less than 13% of total β-galactosidase activity was localized to the supernatants of *V. cholerae* O395-NT, O395-NT (pMOhly1), and O395-NT (pETR14) (Fig. 1B). These data illustrate that the *E. coli* hemolysin secretory pathway can be used in *V. cholerae* to effect secretion of a fusion polypeptide that contains a heterologous antigen fused to a HlyA secretion signal sequence.

To further demonstrate the ability of *V. cholerae* to recognize and export heterologous antigens containing the HlyA export recognition sequence, pETR14 was introduced into a number of *V. cholerae* strains (Table 1 and Fig. 1C). Of the tested strains, *V. cholerae*-O1 classical strain 569B and derivatives of *V. cholerae*-O1 classical strain O395 were the most efficient in exporting the toxin A-HlyA fusion polypeptide to the supernatant fraction. With *V. cholerae*-O139 Bengal 2, approximately 40% of the toxin A-HlyA fusion polypeptide exported to the supernatant. The classical *V. cholerae*-O1 CA401 and El Tor O1 strain Bahrain 2 and C6709 (with its derivative Peru2) were less efficient exporters of the fusion polypeptide.

Intestinal Colonization of O395-NT (pETR14) in Rabbits.

In order to investigate the duration of colonization of rabbit intestines with *V. cholerae* vaccine strains containing pETR14, quantitative cultures of washed rabbit ilea were inoculated on serial days (Figs. 2A and 2B). The *V. cholerae* vector strains containing pETR14 slowly decreased in number over time, but were still recoverable on the last date of examination (day 5) (Fig. 2A). The addition of ampicillin (1 mg/ml) to the water supply caused a marked decrease in the recoverability of both vector *V. cholerae* and pETR14 (Amp^r). On day 2, the number of recoverable *V. cholerae* organisms per gm of tissue in animals treated with ampicillin was a log less than in untreated animals. Almost 100% of the recoverable isolates in the animals contained plasmid pETR14. By day 3, no *V. cholerae* vector or plasmid was recoverable in animals receiving ampicillin-supplemented water, while 10^{3-4} organisms per gram of ileum were still recoverable in rabbits that did not receive ampicillin (Fig. 2B). In sum, pETR14 was

present for at least five days after oral inoculation. In addition, the use of antibiotics is unnecessary to allow the *V. cholerae* to colonize rabbit intestinal surfaces.

Serum Vibriocidal Responses.

As determined by vibriocidal antibody titers measured on days 14 and 28, the oral immunization of rabbits was successful (Fig. 3). In this example, no appreciable booster effect was seen after day 14 reinoculation, as judged by day 28 titers, and no appreciable vibriocidal booster effect was evident in animals that received cholera holotoxin as an immunoadjuvant (Fig. 3).

Serum and Bile Antibody Responses to Toxin A-HlyA Expressed by *V. cholerae*.

Animals inoculated with *V. cholerae* O395-NT expressing the toxin A-HlyA fusion polypeptide from pETR14 produced significant day 21 ($p<0.01$) and day 28 ($p<0.05$) serum IgG anti-*C. difficile* toxin A antibody titers (Fig. 4). The most prominent serum IgG anti-toxin A antibody response was detected in serum of an animal that received O395-NT (pETR14) with cholera toxin as an immunoadjuvant (end geometric mean titer (GMT) 1:2,085). Although a significant serum IgG response was detected, no appreciable serum IgA response was detected (data not shown). The most prominent IgA anti-toxin A antibody responses in bile was seen among those animals that received pETR14 in the presence of cholera toxin (Fig. 5).

Protection from the Action of *C. difficile* Toxin A in the Rabbit Il al L op Challenge Assay.

Responses to PBS/BSA (negative control) and 10 μg of cholera toxin (positive control) were similar among all groups of animals tested (see Figs. 6A and 6D). Examination of intestinal segments into which 1 μg of *C. difficile* toxin A had been instilled revealed significant reductions in weight/length ratios in animals that received 0395-NT (pETR14) either with ($p<0.05$) or without ($p<0.01$) immunoadjuvantative cholera toxin (see Fig. 6B). The level of protection was less marked in intestinal segments that received 5 μg of toxin A, although a mild reduction in the weight/length ratio was detected in animals that received 0395-NT (pETR14) with immunoadjuvantative cholera holotoxin (Fig. 6C). Thus, these results indicate that 0395-NT (pETR14) provides an immunoprotective effect in animals challenged with *C. difficile* toxin A.

Histological examination was performed on intestinal segments from the control animals and from the vaccinated animal that had the most prominent anti-*C. difficile* toxin A serological response prior to challenge. Examination of ileal segments challenged with PBS/BSA revealed normal intestinal architecture in all animals examined (Fig. 7A). Histological examination of ileal segments after challenge with cholera toxin revealed no necrosis or cellular damage; however, there was splaying of intestinal villi in all animals examined, a finding consistent with the marked fluid secretory response induced by cholera toxin (Fig. 7B). Histological results obtained following challenge with *C. difficile* toxin A varied, depending on the dose of toxin A used, and on the vaccination status of the examined animal. Intestinal segments from control animals challenged with toxin A exhibited marked histological changes. In control animals challenged with 1 μg of toxin A, there was severe villous necrosis, with

almost complete loss of villous height. Residual villous structures were markedly edematous and hemorrhagic. Multifocal crypt necrosis was present. The muscularis mucosae was intact, but prominent edema and vascular congestion were present in the submucosa (Fig. 7B). Following challenge with 1 μg of toxin A, intestinal segments from the vaccinated animal exhibited only partial villous necrosis, with preservation of over 50% of villous height. Although crypt areas containing mild architectural distortions were present, no deeper hemorrhagic or necrotic areas were detected (Fig. 7D).

Challenge of control animals with 5 μg of toxin A produced total villous necrosis in ileal loops (Fig. 7C). Edema and hemorrhage were prominent. Extensive crypt necrosis was present with complete loss of crypts focally. Areas of focal necrosis were also present in the muscularis mucosae. The submucosa was markedly edematous, hemorrhagic and necrotic. The muscularis externa contained extensive hemorrhage, and there was pronounced separation of the longitudinal and circular muscular layers by edema and hemorrhage. In contrast, histological examination of intestinal segments following challenge of the vaccinated animal with 5 μg of toxin A reveal only subtotal villous necrosis with villous hemorrhage (Fig. 7E). There were focal areas of crypt hemorrhage and necrosis; other structures were unremarkable. The histological changes observed in intestinal segments of the vaccinated animal following challenge with 5 μg of toxin A were thought to be equivalent to the histological changes observed in the intestinal segments of control animals following challenge with the five-fold lower dose (1 μg) of toxin A. The fact that toxin A-induced intestinal damage was reduced but not completely eliminated in this particular vaccinated animal could be due to the high potency of

even small amounts of this toxin in the ileal loop assay, to suboptimal immunological responses to toxin A in vaccinated animals, or to both possibilities. Nonetheless, the results indicate that large polypeptide epitopes fused to the secretion signal of *E. coli* HlyA can be secreted by *V. cholerae* in the presence of HlyB and HlyD, and the invention can be used to induce protective immunity in mammals against the heterologous antigen *C. difficile* toxin A.

EXAMPLE 2

A second way of introducing a *C. difficile*-derived heterologous antigen into a *V. cholerae* vaccine strain involves generation of a fusion polypeptide containing an epitope derived from *C. difficile* toxin A or toxin B fused to the amino terminus of cholera toxin B subunit (CtxB). An oligonucleotide encoding the epitope (TIDGKKYYFN, SEQ ID NO:1) is synthesized with an *Nhe*I half site added at either end, and is inserted into a CtxB-encoding construct containing an *Nhe*I restriction site just downstream of the sequence encoding the signal sequence cleavage site in CtxB. This leaves the peptide epitope inserted two residues from the amino terminus of mature CtxB. Following transformation, individual transformants are screened by PCR, using primers that generate an approximately 150 bp fragment across the *Nhe*I site in the starting plasmid and looking for the expected increase in size of approximately 30 bp in the correct construct. The plasmids from transformants appearing correct by PCR are confirmed by DNA sequencing. The construct is introduced into *V. cholerae* cells (e.g., strain Peru 2) using *in vivo* marker exchange into *lacZ* of *V. cholerae*. Expression and secretion of the fusion polypeptide can be assessed by immunological techniques,

employing antibodies specific for CtxB and for the *C. difficile* toxin A or toxin B epitope.

EXAMPLE 3

A third way to express a *C. difficile* toxin A or toxin B epitope in *V. cholerae* is by fusing the epitope to the nontoxic A_2 portion of the *V. cholerae* toxin A subunit, $CtxA_2$, and co-expressing this fusion polypeptide with CtxB. The CtxB can be naturally expressed from a chromosome of the cell, or can be encoded by a recombinant plasmid. This fusion is conveniently carried out using a DNA encoding a 44 amino acid segment of the peptide repeat region of *C. difficile* toxin A, which segment is recognized by a commercially available monoclonal antibody PCG-4 (TechLab, Blacksburg, Virginia). The plasmid encodes *ctxA*$_2$ and *ctxB* cloned between unique *Xho*I and *Bpu*1102 I sites. Upstream of the *Xho*I site is a unique *Nco* I site, which is situated at the end of a *pelB* signal sequence. (Any signal sequence which functions in *V. cholerae* can be used.) The heterologous epitope-encoding sequence with *Nco*I and *Xho*I sites at the appropriate ends is inserted between and in frame with the signal sequence coding region and the *ctxA*$_2$ coding region. CtxB is separately translated from its own Shine-Dalgarno sequence in this plasmid. Transformants are screened by PCR and sequenced. The plasmid is then introduced into a *V. cholerae* vaccine strain. Expression is assessed *in vitro* and *in vivo*.

EXAMPLE 4

The *V. cholerae* strains of the invention are useful as vaccines capable of inducing immunity to a heterologous antigen(s) derived from an infectious organism. Because the strains are attenuated (i.e., do not induce a significant toxic reaction in the vaccinee),

they can be used as live-cell vaccines, permitting effective immunity to result from administration of a single dose of the vaccine. An effective oral dose of the vaccine would contain approximately 10^6 to 10^{10} bacteria in a volume of approximately 150 ml liquid. The diluent used would typically be water or an aqueous solution, such as 2 grams of sodium bicarbonate dissolved in 150 ml distilled water, which may be ingested by the vaccinee at one sitting, either all at once or over any convenient period of time.

What is claimed is:

1. A *V. cholerae* cell containing DNA encoding:

(*A*) *E. coli* HlyB,

(*B*) *E. coli* HlyD, and

(*C*) a fusion polypeptide, wherein the fusion polypeptide comprises:

(*i*) an antigenic part, or all, of a heterologous antigenic polypeptide; and

(*ii*) an *E. coli* HlyA secretion signal sequence.

2. The *V. cholerae* cell of claim 1, wherein the heterologous antigenic polypeptide has the sequence of a polypeptide that is naturally expressed by an infectious organism.

3. The *V. cholerae* cell of claim 2, wherein the infectious organism is a bacterium.

4. The *V. cholerae* cell of claim 1, wherein the heterologous antigenic polypeptide is an immunogenic bacterial toxin.

5. The *V. cholerae* cell of claim 4, wherein the bacterial toxin is *C. difficile* toxin A or *C. difficile* toxin B.

6. The *V. cholerae* cell of claim 4, wherein the bacterial toxin is selected from the group consisting of Shiga toxin, diphtheria toxin, *Pseudomonas* exotoxin A, pertussis toxin, tetanus toxin, anthrax toxin, *E. coli* LT, *E. coli* ST, and *E. coli* Shiga-like toxin.

7. The *V. cholerae* cell of claim 3, wherein the heterologous antigenic polypeptide is selected from the group consisting of a colonization factor of

diarrheogenic *E. coli*, a colonization factor of *Bordetella pertussis*, a pilin of uropathogenic *E. coli*, and a pilin of *Neisseria gonorrhoeae*.

8. The *V. cholerae* cell of claim 5, wherein the bacterial toxin is *C. difficile* toxin A and the fusion polypeptide comprises an antigenic part of the carboxy terminal third of *C. difficile* toxin A.

9. The *V. cholerae* cell of claim 1, wherein the heterologous antigenic polypeptide is an immunogenic viral surface protein.

10. The *V. cholerae* cell of claim 9, wherein the virus is a human immunodeficiency virus, a Herpes virus, an influenza virus, a poliomyelitis virus, a measles virus, a mumps virus, a rubella virus, a rotavirus, a respiratory syncytial virus, an adenovirus, or a papilloma virus.

11. The *V. cholerae* cell of claim 1, wherein the *V. cholerae* cell does not express biologically active cholera toxin A subunit.

12. The *V. cholerae* cell of claim 1, wherein the background strain of the *V. cholerae* cell is selected from the group consisting of *V. cholerae*-01 strain 569B, *V. cholerae*-01 strain 0395, and *V. cholerae*-0139 strain Bengal 2.

13. A method for inducing in an animal an immune response against a heterologous antigen, the method comprising administering to the animal a recombinant *V. cholerae* cell containing DNA enccding:

(*A*) *E. coli* HlyB,

(*B*) *E. coli* HlyD, and

(*C*) a fusion polypeptide, wherein the fusion polypeptide comprises:

(*i*) an antigenic part, or all, of an heterologous antigenic polypeptide; and

(*ii*) an *E. coli* HlyA secretion signal sequence.

14. The method of claim 13, wherein the heterologous antigenic polypeptide has the sequence of a polypeptide that is naturally expressed by an infectious organism.

15. The method of claim 14, wherein the infectious organism is a bacterium.

16. The method of claim 13, wherein the heterologous antigenic polypeptide is an immunogenic bacterial protein.

17. The method of claim 16, wherein the bacterial protein is *C. difficile* toxin A or toxin B.

18. The method of claim 17, wherein the bacterial protein is selected from the group consisting of Shiga toxin, diphtheria toxin, *Pseudomonas* exotoxin A, pertussis toxin, tetanus toxin, anthrax toxin, *E. coli* LT, *E. coli* ST, and *E. coli* Shiga-like toxin.

19. The method of claim 16, wherein the bacterial protein is selected from the group consisting of a colonization factor of diarrheogenic *E. coli*, a colonization factor of *Bordetella pertussis*, a pilin of

uropathogenic *E. coli*, and a pilin of *Neisseria gonorrhoeae*.

20. The method of claim 17, wherein the bacterial protein is *C. difficile* toxin A and the fusion polypeptide comprises an antigenic part of the carboxy terminal third of *C. difficile* toxin A.

21. The method of claim 13, wherein the heterologous antigenic polypeptide is a viral surface protein.

22. The method of claim 21, wherein the virus is a human immunodeficiency virus, a Herpes virus, an influenza virus, a poliomyelitis virus, a measles virus, a mumps virus, a rubella virus, a rotavirus, a respiratory syncytial virus, an adenovirus, or a papilloma virus.

23. The method of claim 13, wherein the background strain of the *V. cholerae* cell is selected from the group consisting of *V. cholerae*-01 strain 569B, *V. cholerae*-01 strain 0395, and *V. cholerae*-0139 strain Bengal 2.

24. A *V. cholerae* cell containing DNA encoding a fusion polypeptide, wherein the fusion polypeptide comprises:

(*A*) an antigenic fragment of *C. difficile* toxin A or toxin B fused to

(*B*) the N-terminus of cholera toxin B subunit.

25. The *V. cholerae* cell of claim 24, wherein the amino acid sequence of the antigenic fragment consists essentially of TIDGKKYYFN (SEQ ID NO:1).

26. The *V. cholerae* cell of claim 24, wherein the background strain of the *V. cholerae* cell is selected from the group consisting of *V. cholerae*-O1 strain 569B, *V. cholerae*-01 strain 0395, and *V. cholerae*-0139 strain Bengal 2.

27. A method for inducing in an animal an immune response against *C. difficile* toxin A or toxin B, the method comprising administering to the animal a recombinant *V. cholerae* cell containing DNA encoding a fusion polypeptide, wherein the fusion polypeptide comprises:

(*A*) an antigenic fragment of *C. difficile* toxin A or toxin B fused to

(*B*) the N-terminus of cholera toxin B subunit, the fusion polypeptide being expressed from said DNA in the animal.

28. The method of claim 27, wherein the background strain of the *V. cholerae* cell is selected from the group consisting of *V. cholerae*-O1 strain 569B, *V. cholerae*-01 strain 0395, and *V. cholerae*-0139 strain Bengal 2.

29. A *V. cholerae* cell containing DNA encoding:

(*A*) cholera toxin B subunit, the DNA encoding the cholera toxin B subunit being located on a chromosome or a plasmid, and

(*B*) a fusion polypeptide, the fusion polypeptide comprising (*i*) a secretion signal sequence, (*ii*) a

heterologous antigenic polypeptide, and (*iii*) cholera toxin A2 subunit.

30. The *V. cholerae* cell of claim 29, wherein the heterologous antigenic polypeptide is an antigenic portion or all of a bacterial protein.

31. The *V. cholerae* cell of claim 30, wherein the bacterial protein is *C. difficile* toxin A or toxin B.

32. The *V. cholerae* cell of claim 30, wherein the bacterial protein is selected from the group consisting of toxin A, Shiga toxin, diphtheria toxin, *Pseudomonas* exotoxin A, pertussis toxin, tetanus toxin, anthrax toxin, *E. coli* LT, *E. coli* ST, and *E. coli* Shiga-like toxin.

33. The *V. cholerae* cell of claim 31, wherein the bacterial protein is *C. difficile* toxin A and the antigenic portion consists of about 20-100 amino acid residues comprising at least one intact repeat of the *C. difficile* toxin A repeating peptide sequence.

34. The *V. cholerae* cell of claim 29, wherein the heterologous antigenic polypeptide is a viral surface protein.

35. The *V. cholerae* cell of claim 34, wherein the virus is a human immunodeficiency virus, a Herpes virus, an influenza virus, a poliomyelitis virus, a measles virus, a mumps virus, a rubella virus, a rotavirus, a respiratory syncytial virus, an adenovirus, or a papilloma virus.

36. The *V. cholerae* cell of claim 29, wherein the secretion signal sequence is a non-*V. cholerae* signal sequence.

37. The *V. cholerae* cell of claim 29, wherein the background strain of the *V. cholerae* cell is selected from the group consisting of *V. cholerae*-01 strain 569B, *V. cholerae*-01 strain 0395, and *V. cholerae*-0139 strain Bengal 2.

38. A method for inducing in an animal an immune response against a heterologous antigen, the process comprising administering to the animal a recombinant *V. cholerae* cell containing DNA encoding:

(*A*) cholera toxin B subunit, the DNA encoding the cholera toxin B subunit being located on a chromosome or a plasmid, and

(*B*) a fusion polypeptide, wherein the fusion polypeptide comprises (*i*) a secretion signal sequence, (*ii*) a heterologous antigenic polypeptide, and (*iii*) cholera toxin A2 subunit.

39. The method of claim 38, wherein the heterologous antigenic polypeptide is an antigenic portion or all of a bacterial protein.

40. The method of claim 39, wherein the bacterial protein is *C. difficile* toxin A or toxin B.

41. The method of claim 39, wherein the bacterial protein is selected from the group consisting of Shiga toxin, diphtheria toxin, *Pseudomonas* exotoxin A, pertussis toxin, tetanus toxin, anthrax toxin, *E. coli* LT, *E. coli* ST, and *E. coli* Shiga-like toxin.

42. The method of claim 38, wherein the heterologous antigenic polypeptide consists of about 20-100 amino acid residues of *C. difficile* toxin A comprising at least one intact repeat of the *C. difficile* toxin A repeating peptide sequence.

43. The method of claim 38, wherein the heterologous antigenic polypeptide is a viral surface protein.

44. The method of claim 43, wherein the virus is a human immunodeficiency virus, a Herpes virus, an influenza virus, a poliomyelitis virus, a measles virus, a mumps virus, a rubella virus, a rotavirus, a respiratory syncytial virus, an adenovirus, or a papilloma virus.

45. The method of claim 38, wherein the background strain of the *V. cholerae* cell is selected from the group consisting of *V. cholerae*-01 strain 569B, *V. cholerae*-01 strain 0395, and *V. cholerae*-0139 strain Bengal 2.

46. The *V. cholerae* cell of claim 1, wherein the DNA further encodes a detoxified immunoadjuvant.

47. The *V. cholerae* cell of claim 46, wherein the detoxified immunoadjuvant is detoxified cholera toxin or detoxified heat labile enterotoxin.

48. The cell of claim 24, wherein the DNA further encodes a detoxified immunoadjuvant.

49. The cell of claim 29, wherein the DNA further encodes a detoxified immunoadjuvant.

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 97/21359

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/74 C12N15/62 C12N15/31 C12N1/21 C07K14/33
C07K14/28 C07K14/245 C07K16/12 A61K39/08 A61K39/106
//(C12N1/21,C12R1:63)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	RYAN E.T. ET AL.: "Protective immunity against C. difficile Toxin A induced by oral immunization with a live attenuated V. cholerae vector strain" INFECTION & IMMUNITY, vol. 65, no. 7, July 1997, pages 2941-2949, XP002060318 see the whole document	1-23
Y	WO 94 19482 A (GEN HOSPITAL CORP ;HARVARD COLLEGE (US)) 1 September 1994 see abstract see page 3, line 18 - page 5, line 23 see page 8, line 9 - line 23 see claims 1,2	1-4,6,7, 9,10
A		5,8, 11-23, 29-47,49

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 March 1998	21.04.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte tonal Application No
PCT/US 97/21359

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TZSCHASCHEL B.D. ET AL.: "An E. coli hemolysin transport system-based vector for the export of polypeptides: Export of Shiga-like toxin IIeB subunit by S. typhimurium AroA" NATURE BIOTECHNOLOGY, vol. 14, 14 June 1996, pages 765-769, XP002060319 see abstract see figures 1,2	1-4,6,7, 9,10
A	---	5,8, 11-23, 46,47
A	WO 96 12802 A (OPHIDIAN PHARM INC ;WILLIAMS JAMES A (US); PADHYE NISHA V (US); KI) 2 May 1996 see abstract see claims 36-129	5,8,17, 20, 24-28, 31,33, 40,42,48
A	--- EP 0 125 228 A (HARVARD COLLEGE) 14 November 1984 cited in the application see abstract see figures 1,2	11,12, 23,26, 28-45,49
A	--- WO 93 13202 A (SCLAVO BIOCINE SPA) 8 July 1993 see abstract	46-49
A	--- WO 91 07979 A (INNOVATIVE TECH CENTER) 13 June 1991 see abstract see page 12, line 23 - page 15, line 2	24-28,48

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int Jonal Application No
PCT/US 97/21359

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9419482 A	01-09-94	AU 683454 B	13-11-97
		AU 6244594 A	14-09-94
		CA 2156191 A	01-09-94
		EP 0692031 A	17-01-96
		JP 8506963 T	30-07-96
WO 9612802 A	02-05-96	US 5601823 A	11-02-97
		US 5196193 A	23-03-93
		AU 3968395 A	15-05-96
		CA 2203504 A	02-05-96
		EP 0796326 A	24-09-97
		FI 971732 A	23-06-97
		NO 971868 A	24-06-97
		PL 320214 A	15-09-97
		ZA 9508990 A	15-05-96
		AU 6653894 A	04-07-94
		CA 2150935 A	23-06-94
		EP 0671902 A	20-09-95
		WO 9413264 A	23-06-94
		US 5466672 A	14-11-95
		US 5599539 A	04-02-97
		US 5719267 A	17-02-98
		AU 638786 B	08-07-93
		AU 6895191 A	31-05-91
		EP 0498854 A	19-08-92
		WO 9106306 A	16-05-91
		US 5443976 A	22-08-95
		US 5340923 A	23-08-94
EP 0125228 A	14-11-84	US 4882278 A	21-11-89
		AU 585481 B	22-06-89
		AU 2727084 A	01-11-84
		CA 1326218 A	18-01-94
		DE 3472114 A	21-07-88
		DK 213784 A	30-10-84
		EG 17879 A	30-08-91
		JP 2012452 C	02-02-96
		JP 7040921 B	10-05-95
		JP 60037980 A	27-02-85
		PH 25301 A	30-04-91

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/US 97/21359

Box I Observations where certain claims were f und unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INF RMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 13-23, 27-28 and 38-45 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/21359

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9313202 A	08-07-93	IT 1253009 B	10-07-95
		AU 3347693 A	28-07-93
		CA 2127091 A	08-07-93
		EP 0620850 A	26-10-94
		JP 7506240 T	13-07-95
		MX 9207685 A	31-05-94
WO 9107979 A	13-06-91	CA 2069106 A	30-05-91
		EP 0502099 A	09-09-92
		JP 5503420 T	10-06-93

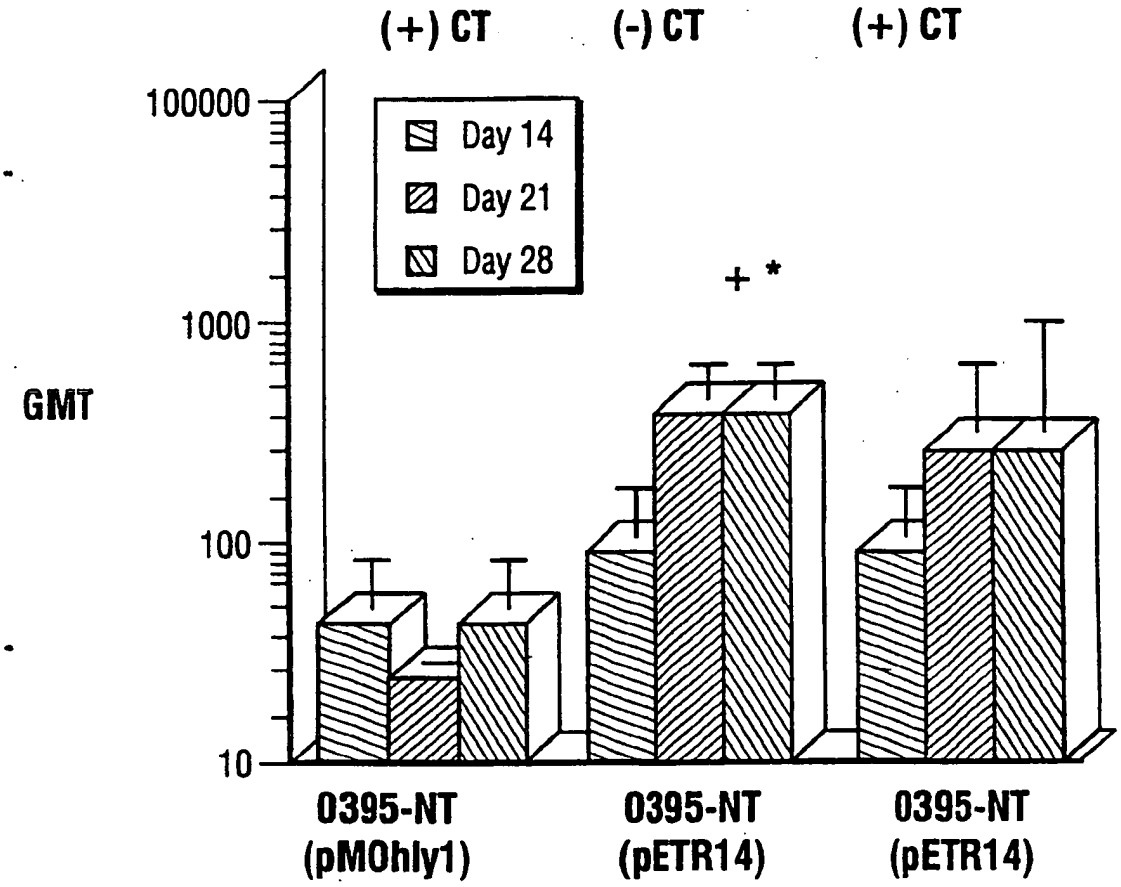

(+) CT
(-) CT
(+) CT
100000
1000
100
10
GMT
Day 14
Day 21
Day 28
+ *
0395-NT
(pMOhly1)
0395-NT
(pETR14)
0395-NT
(pETR14)

Figure 4

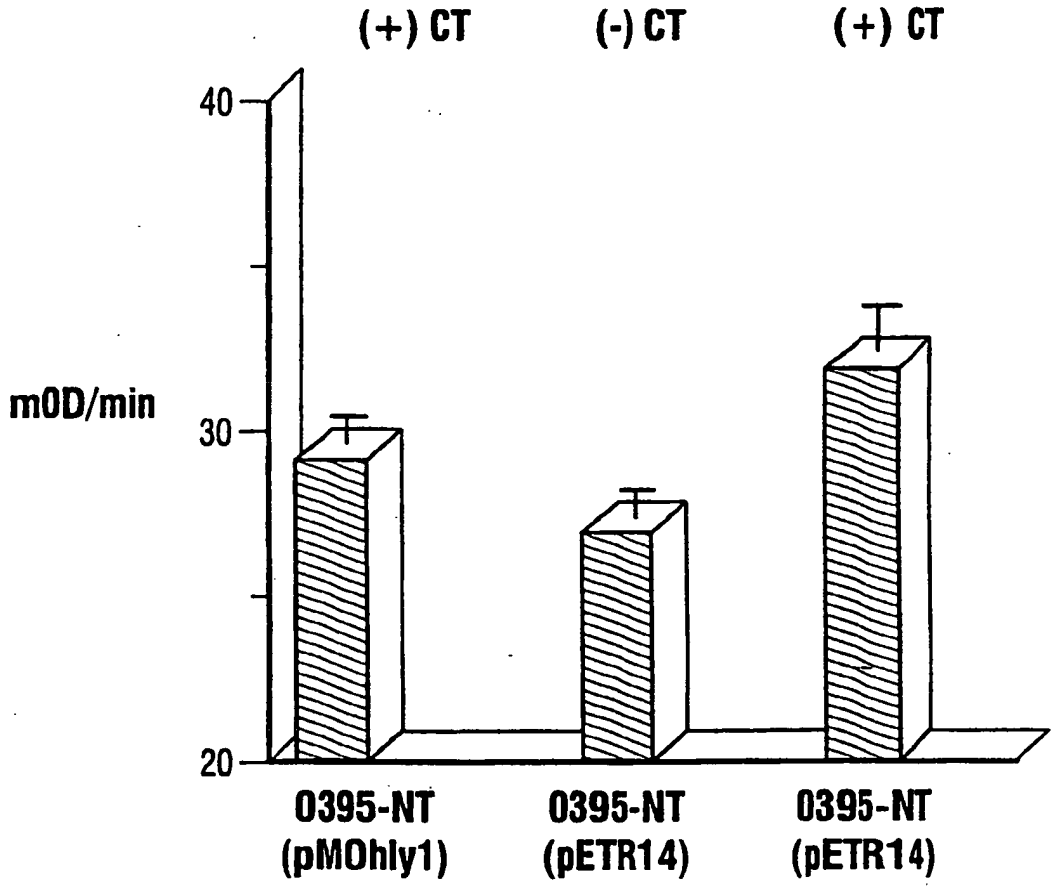

(+) CT
(-) CT
(+) CT
40
30
20
mOD/min
0395-NT
(pMOhly1)
0395-NT
(pETR14)
0395-NT
(pETR14)

Figure 5

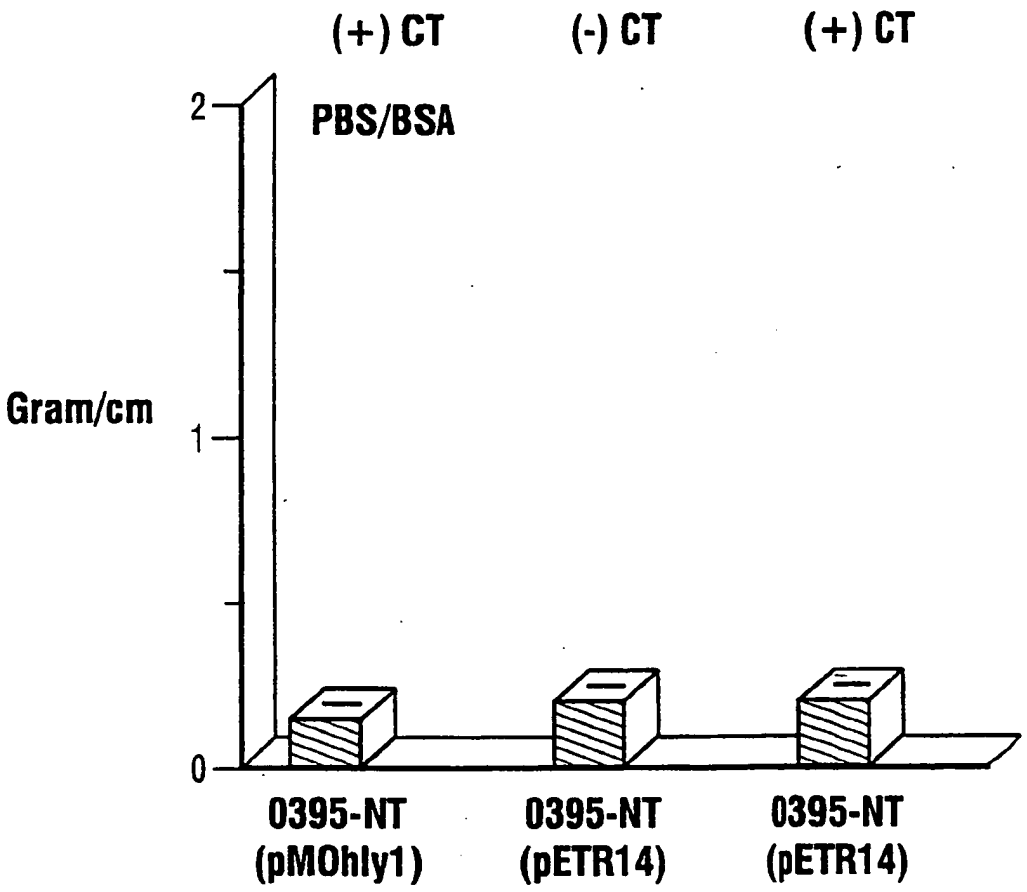

(+) CT
(-) CT
(+) CT
PBS/BSA
Gram/cm
2
1
0
0395-NT (pMOhly1)
0395-NT (pETR14)
0395-NT (pETR14)

Figure 6A

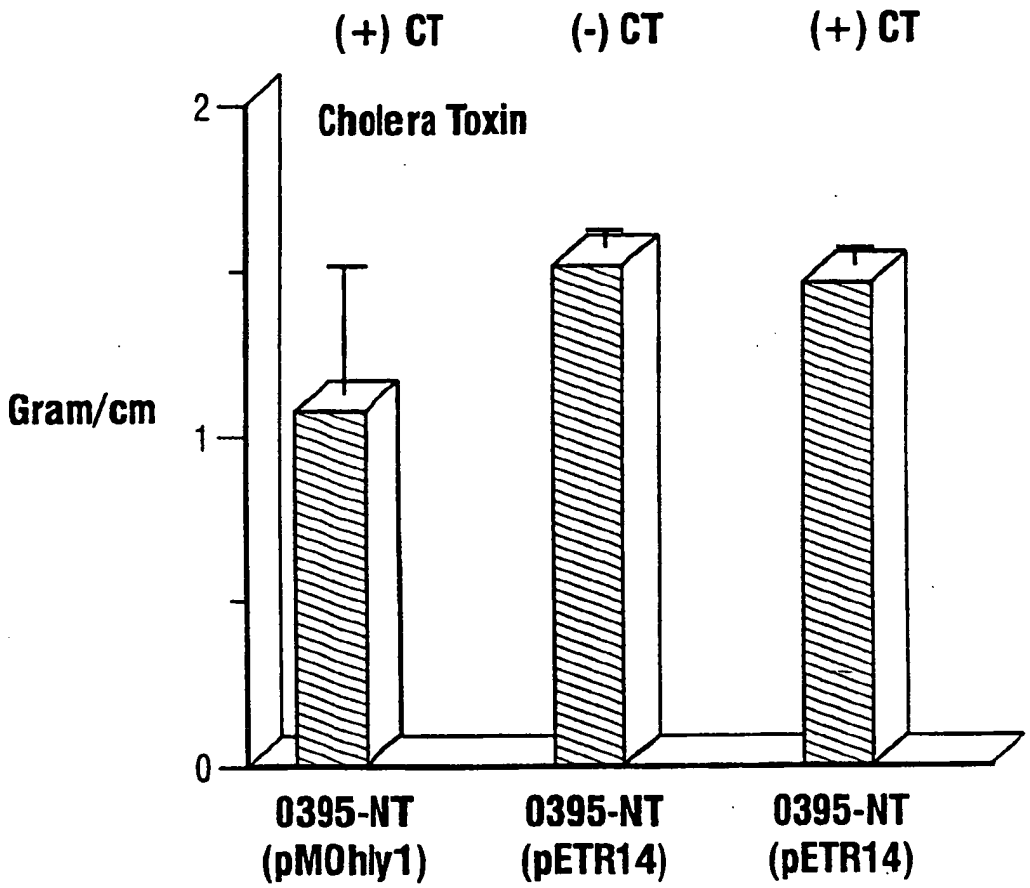

(+) CT
(-) CT
(+) CT
Cholera Toxin
Gram/cm
2
1
0
0395-NT
(pMOhly1)
0395-NT
(pETR14)
0395-NT
(pETR14)

Figure 6B

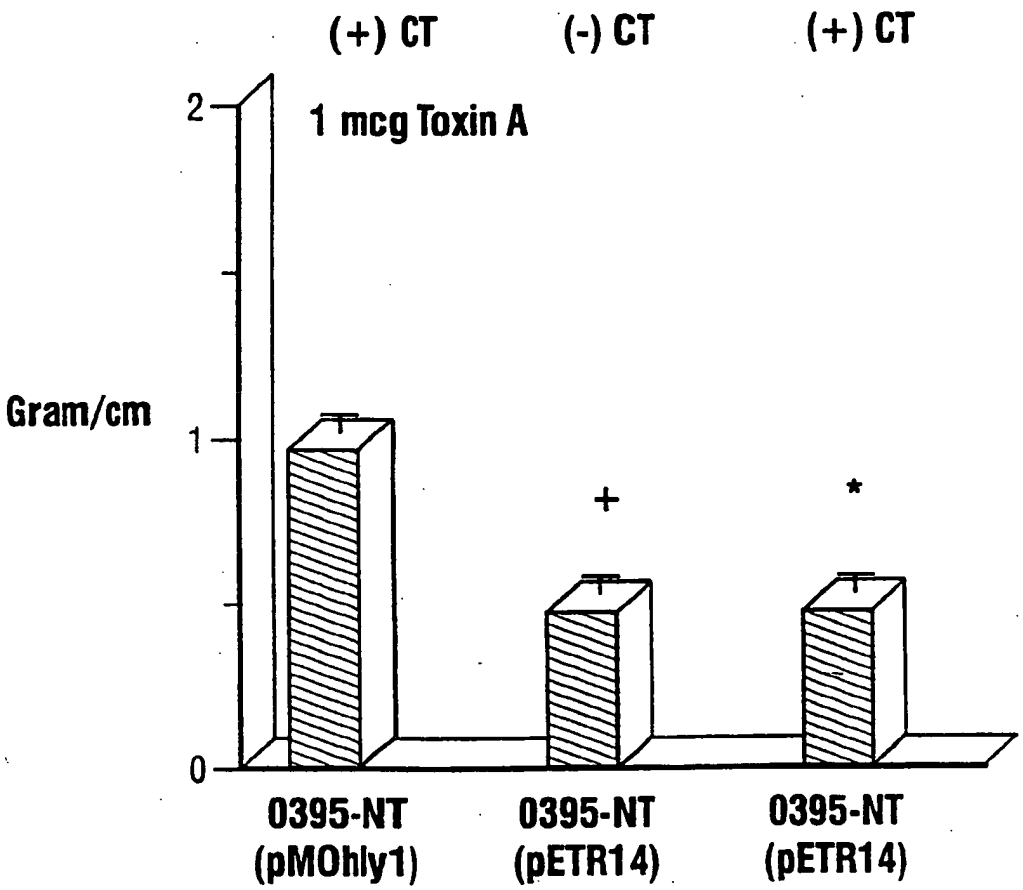

(+) CT
(-) CT
(+) CT
1 mcg Toxin A
Gram/cm
2
1
0
+
*
0395-NT
(pMOhly1)
0395-NT
(pETR14)
0395-NT
(pETR14)

Figure 6C

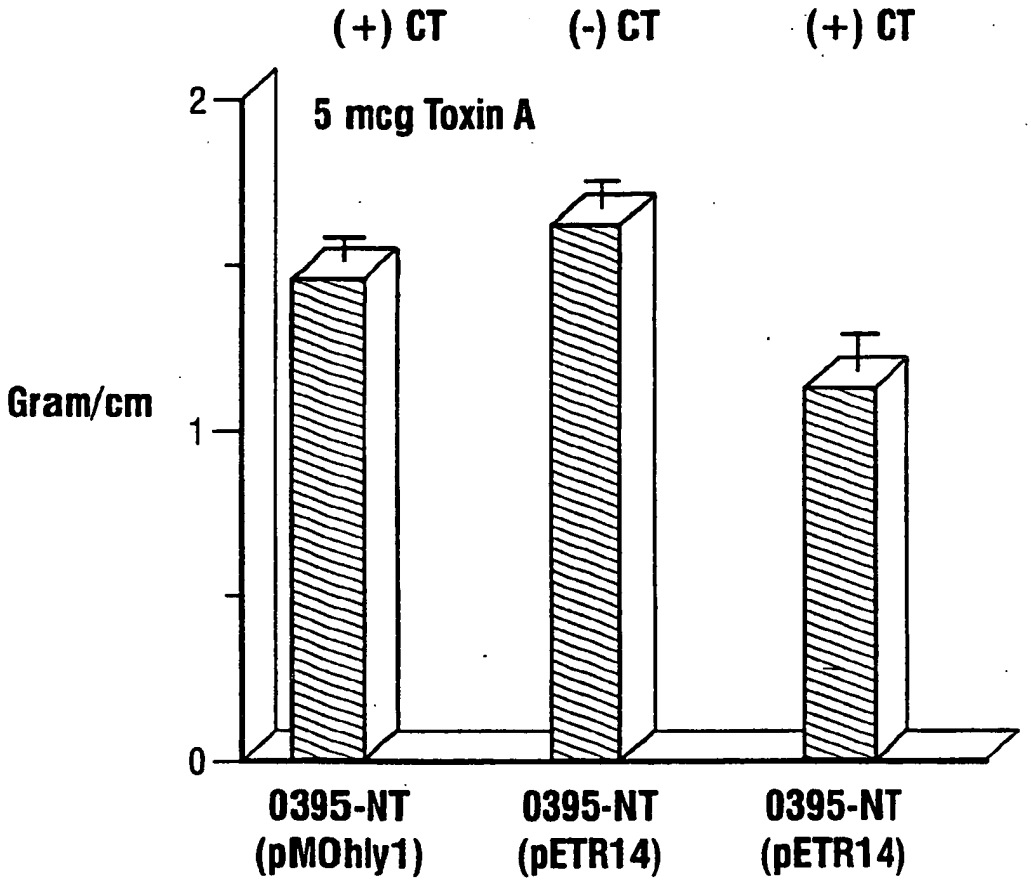
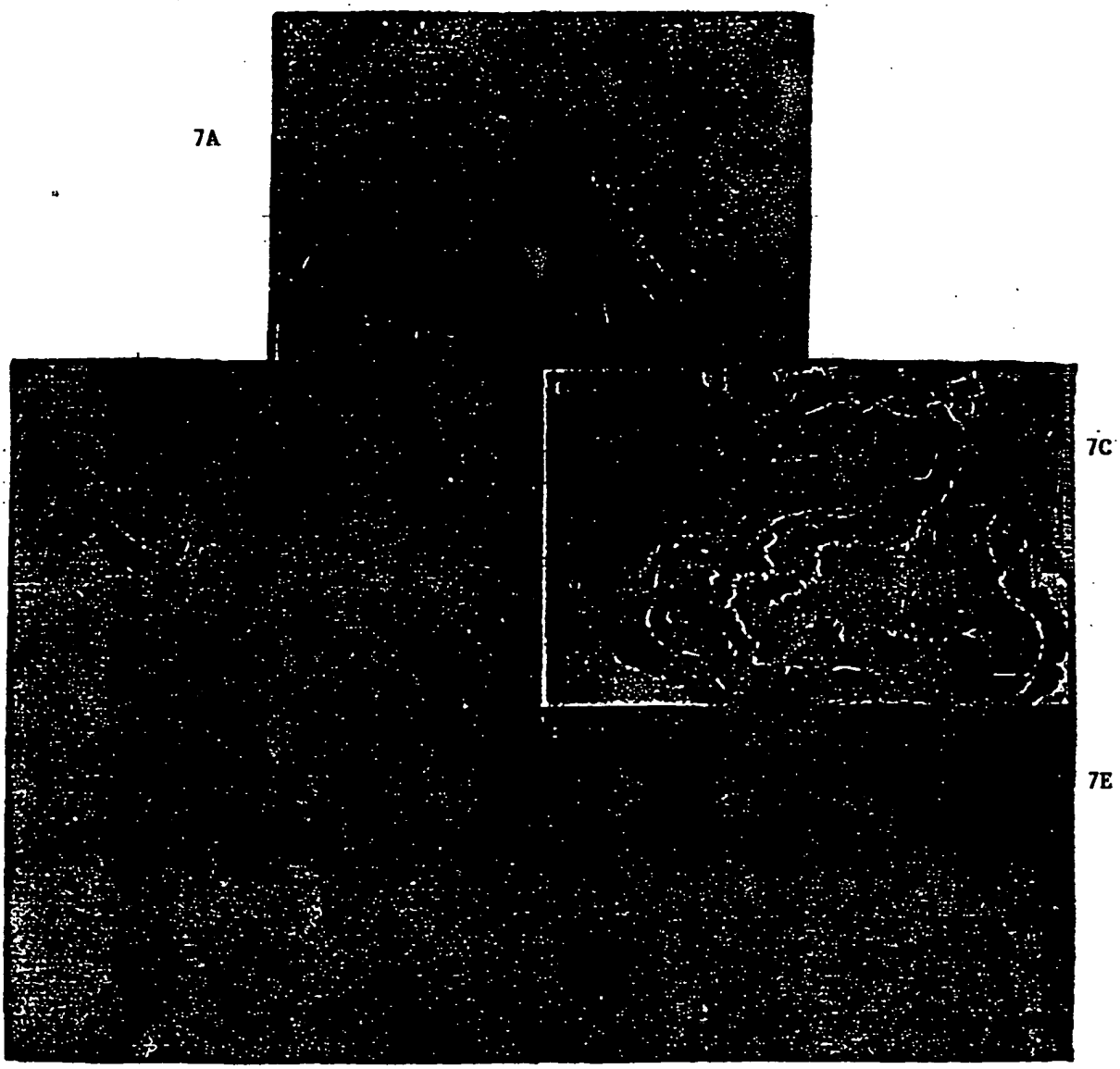

(+) CT
(-) CT
(+) CT
5 mcg Toxin A
Gram/cm
2
1
0
0395-NT (pMOhly1)
0395-NT (pETR14)
0395-NT (pETR14)

Figure 6D

Figs. 7A-7E



INTERNATIONAL SEARCH REPORT

Intr ional Application No
PCT/US 97/21359

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/74 C12N15/62 C12N15/31 C12N1/21 C07K14/33
C07K14/28 C07K14/245 C07K16/12 A61K39/08 A61K39/106
//(C12N1/21,C12R1:63)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	RYAN E.T. ET AL.: "Protective immunity against C. difficile Toxin A induced by oral immunization with a live attenuated V. cholerae vector strain" INFECTION & IMMUNITY, vol. 65, no. 7, July 1997, pages 2941-2949, XP002060318 see the whole document ---	1-23
Y	WO 94 19482 A (GEN HOSPITAL CORP ;HARVARD COLLEGE (US)) 1 September 1994 see abstract see page 3, line 18 - page 5, line 23 see page 8, line 9 - line 23 see claims 1,2	1-4,6,7, 9,10
A	--- -/--	5,8, 11-23, 29-47,49

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 March 1998	21.04.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Galli, I

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 97/21359

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TZSCHASCHEL B.D. ET AL.: "An E. coli hemolysin transport system-based vector for the export of polypeptides: Export of Shiga-like toxin IIeB subunit by S. typhimurium AroA" NATURE BIOTECHNOLOGY, vol. 14, 14 June 1996, pages 765-769, XP002060319 see abstract see figures 1,2	1-4,6,7, 9,10
A	---	5,8, 11-23, 46,47
A	WO 96 12802 A (OPHIDIAN PHARM INC ;WILLIAMS JAMES A (US); PADHYE NISHA V (US); KI) 2 May 1996 see abstract see claims 36-129	5,8,17, 20, 24-28, 31,33, 40,42,48
A	--- EP 0 125 228 A (HARVARD COLLEGE) 14 November 1984 cited in the application see abstract see figures 1,2	11,12, 23,26, 28-45,49
A	--- WO 93 13202 A (SCLAVO BIOCINE SPA) 8 July 1993 see abstract	46-49
A	--- WO 91 07979 A (INNOVATIVE TECH CENTER) 13 June 1991 see abstract see page 12, line 23 - page 15, line 2	24-28,48

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/21359

Box I bservations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remarkon Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 13-23, 27-28 and 38-45 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int Jonal Application No
PCT/US 97/21359

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9419482 A	01-09-94	AU 683454 B	13-11-97
		AU 6244594 A	14-09-94
		CA 2156191 A	01-09-94
		EP 0692031 A	17-01-96
		JP 8506963 T	30-07-96
WO 9612802 A	02-05-96	US 5601823 A	11-02-97
		US 5196193 A	23-03-93
		AU 3968395 A	15-05-96
		CA 2203504 A	02-05-96
		EP 0796326 A	24-09-97
		FI 971732 A	23-06-97
		NO 971868 A	24-06-97
		PL 320214 A	15-09-97
		ZA 9508990 A	15-05-96
		AU 6653894 A	04-07-94
		CA 2150935 A	23-06-94
		EP 0671902 A	20-09-95
		WO 9413264 A	23-06-94
		US 5466672 A	14-11-95
		US 5599539 A	04-02-97
		US 5719267 A	17-02-98
		AU 638786 B	08-07-93
		AU 6895191 A	31-05-91
		EP 0498854 A	19-08-92
		WO 9106306 A	16-05-91
		US 5443976 A	22-08-95
		US 5340923 A	23-08-94
EP 0125228 A	14-11-84	US 4882278 A	21-11-89
		AU 585481 B	22-06-89
		AU 2727084 A	01-11-84
		CA 1326218 A	18-01-94
		DE 3472114 A	21-07-88
		DK 213784 A	30-10-84
		EG 17879 A	30-08-91
		JP 2012452 C	02-02-96
		JP 7040921 B	10-05-95
		JP 60037980 A	27-02-85
		PH 25301 A	30-04-91

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/21359

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9313202 A	08-07-93	IT 1253009 B	10-07-95
		AU 3347693 A	28-07-93
		CA 2127091 A	08-07-93
		EP 0620850 A	26-10-94
		JP 7506240 T	13-07-95
		MX 9207685 A	31-05-94
WO 9107979 A	13-06-91	CA 2069106 A	30-05-91
		EP 0502099 A	09-09-92
		JP 5503420 T	10-06-93